Facilitated transport of Mn^{2+} in sycamore (*Acer pseudoplatanus*) cells and excised maize root tips

A comparative ^{31}P n.m.r. study *in vivo*

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Movement of paramagnetic Mn^{2+} into sycamore (*Acer pseudoplatanus*) cells has been indirectly examined by observing the line broadening exhibited in its ^{31}P n.m.r. spectra. Mn^{2+} was observed to pass into the vacuole, while exhibiting a very minor accumulation in the cytoplasm. With time, gradual leakage of phosphate from the vacuole to the cytoplasm was observed along with an increase in glucose-6-phosphate. Anoxia did not appear to affect the relative distribution of Mn^{2+} in the cytoplasm and vacuole. Under hypoxic conditions restriction of almost all movement of Mn^{2+} across the plasmalemma as well as the tonoplast was observed. In contrast, maize root tips showed entry and complete complexation of nucleotide triphosphate by Mn^{2+} during hypoxia. The rate of passage of Mn^{2+} across the tonoplast in both sycamore and maize root cells is approximately the same. However, the rates of facilitated movement across the respective plasma membranes appear to differ. More rapid movement of Mn^{2+} across the plasmalemma in maize root tip cells allows a gradual build-up of metal ion in the cytoplasm prior to its diffusion across the tonoplast. Sycamore cells undergo a slower uptake of Mn^{2+} into their cytoplasms (comparable with the rate of diffusion through the tonoplast), so little or no observable accumulation of Mn^{2+} is observed in this compartment.

INTRODUCTION

Manganese toxicity is an important growth factor for plants in acid soils (Foy, 1984). Excess Mn^{2+} within plant cell lowers ATP levels and respiration rates (Sirkar & Amin, 1979) as well as activities of enzymes and hormones (Helyar, 1978; Heenan & Campbell, 1981). Tolerance of some plants to manganese has been associated with the oxidizing powers of the plant roots to produce non-toxic MnO_2 (Aoba *et al.*, 1977), or alternatively, rapid absorption and translocation of Mn^{2+} to non-metabolic compartments for entrapment (Foy, 1973).

N.m.r. studies have demonstrated that Mn, because of its paramagnetic properties, can be used as an indirect probe to observe the relaxation of water resonances (Bacic & Ratkovic, 1984) and line broadening of resonances associated with compartmented phosphorus metabolites in excised maize (*Zea mays* L.) root tips (Kime *et al.*, 1982; Pfeffer *et al.*, 1986). Whereas the former studies demonstrated differential relaxation behaviour characteristic of a multiphase uptake of paramagnetic ions, the latter revealed a progressive Mn^{2+} movement into the cytoplasm and vacuole and only reversible efflux across the plasmalemma. Although these results indicated that Mn^{2+} migration could be observed across the plasmalemma under both aerobic and hypoxic conditions, it was not clear, because of the inherent heterogeneity of the tissue and or possible disruption caused by excision, whether these phenomena were characteristic of the entire cell population.

Isolated plant cells and cell cultures offer a unique opportunity to examine the ^{31}P n.m.r. spectra of simple, intact, homogeneous cell populations which can aid in the interpretation of those spectra obtained from a wide range of cell types as found in excised plant tissues. Thus far several research groups have studied such phenomena as phosphorus uptake and storage (Rébeillé *et al.*, 1982), flux (Rébeillé *et al.*, 1983) and intracellular pH (Martin *et al.*, 1982) in sycamore (*Acer pseudoplatanus* L.) cells as well as phosphate status in barley (*Hordeum vulgare* L.) leaf cells (Foyer & Spencer, 1986) and P_i uptake in NaCl-tolerant citrus (*Citrus sinensis* L. Osbeck) cells in aerated solution (Gozal & Navon, 1985). In general, spectra obtained from these preparations were of poorer quality than those obtained from tissue, since continuous liquid perfusion could not be conveniently performed with small cells. However, a recent advance in the design of a modified perfusion system (Hughes *et al.*, 1983; Roby *et al.*, 1987) has improved the spectral resolution and cell viability for long-term experiments, making these experiments comparable with those of their plant tissue counterparts (Loughman & Ratcliffe, 1984).

The present report compares the process of Mn^{2+} transport as observed by ^{31}P n.m.r. in excised maize root tips and sycamore cells, under aerobic and hypoxic conditions.

EXPERIMENTAL

Plant material

The strain of sycamore (*A. pseudoplatanus* L.) used in the current study was generously provided by J. Guern (Plant Cellular Physiology, Gif-sur-Yvette, France). The

Abbreviations used: NDP, nucleotide diphosphate; NTP, nucleotide triphosphate; UDPG, uridine 5′-diphosphoglucose.
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nutrient medium was prepared as described previously (Roby *et al.*, 1987), but Mn^{2+} was excluded to prevent excessive broadening of the ^{31}P orthophosphate vacuolar resonance. The cell suspensions were maintained in exponential growth by frequent subcultures. The medium was adjusted to pH 6.5 to prevent massive influx of P_i into the vacuolar space (Roby *et al.*, 1987).

Root tips (~ 700–900) were excised from 3-day-old seedlings (*Z. mays* L. var. FRB-73) grown at 28 °C in a growth chamber over damp paper for 72 h as described earlier (Pfeffer *et al.*, 1986). Immediately after cutting, the 3–4 mm tips were placed in cold neutral solution (30 ml) containing 0.1 mM-$CaSO_4$ and aerated with O_2 before examination by ^{31}P n.m.r.

^{31}P n.m.r. experiments

^{31}P n.m.r. spectra of sycamore cells (*A. pseudoplatanus* L.) were obtained with a Brüker WM 200 n.m.r. spectrometer operating at 81.01 MHz for ^{31}P. The spectra were obtained with compressed cells (4 cm in height) placed in a 25 mm n.m.r. tube under constant perfusion. Details of this assembly and its operation have been described (Roby *et al.*, 1987). The perfusate consisted of the previously reported culture medium, devoid of phosphate and Mn, saturated with O_2 and adjusted to pH 6.5 (Rébeillé *et al.*, 1985). During anoxia all circulation of the perfusate was stopped.

Spectra were recorded *in vivo* after 3000–6000 accumulations with a repetition rate of 0.6 s. Doubling of the repetition time to 1.2 s showed no appreciable change in the relative intensities of the P_i to nucleotide resonances. The positions of the P_i and glucose-6-phosphate peaks were estimated in 5 mM-KOH/K_2HPO_4 buffers at given pH values. Assignments of the nucleotide and phosphate ester peaks and P_i (cytoplasm and vacuole respectively) were made in accordance with those previously given (Roby *et al.*, 1987).

A reference capillary approx. 0.8 mm in diameter containing 50 mM-methylenediphosphonate (pH 8.9 in 30 mM-Tris) was inserted inside the inner circulating tube (inlet tube) of the n.m.r. tube assembly. The resonance of this reference was assigned a value of 13.68 δ relative to the 85% H_3PO_4 resonance which has a chemical shift of 0.00 δ.

^{31}P n.m.r. spectra of 3–5 mm excised root tips (700–900) were obtained on a narrow bore (54 mm) JEOL GX-400 n.m.r. spectrometer operating at 161.7 MHz. The samples were continually perfused with a recycling perfusion system in a 10 mm n.m.r. tube as previously described (Pfeffer *et al.*, 1986). The circulating perfusate consisted of a 1 litre solution containing 50 mM-glucose, 20 mM-Mes buffer and 0.1 mM-$CaSO_4$ saturated with O_2 and adjusted to pH 6.0. (During hypoxia, the perfusion system circulated the above solution saturated with N_2 gas and only trace amounts of O_2 were present.) A reference capillary containing 120 mM-hexamethylphosphoramide was used to give a satisfactory reference peak for each spectrum. Hexamethylphosphoramide exhibited a resonance at 30.73 δ downfield from 85% H_3PO_4. All chemical shift scales were referenced relative to 85% H_3PO_4 which was assigned to a value of 0.0 δ. Fast acquisition accumulation parameters; 30° pulse (12 μs), 2000 data points zero filled to 16000, recycling time of 0.162 s, 10000–40000 transients/spectrum, 16 kHz spectral widths and 15 Hz line broadening were used for all experiments. Appropriate response factors were established from slow acquisition accumulation experiments to evaluate the interconversion of different components having dissimilar relaxation times. Assignment of the nucleotide, organophosphates and nucleotide shifts were made in accordance with those previously described (Roberts & Jardetzky, 1981; Loughman & Ratcliffe, 1984).

Estimates of intracellular pH from cytoplasmic and vacuolar phosphate were made using the standard reference curve of pH versus ^{31}P chemical shifts (Pfeffer *et al.*, 1986). Solutions prepared for this profile contained 5.0 mM-K_2HPO_4, 2.0 mM-$MgCl_2$ and 100 mM-KCl. All shift references were made with respect to hexamethylphosphoramide at 30.73 δ. ^{31}P line broadening experiments were conducted at pH 7.5 and 5.5 with 10 mM-Hepes and acetate buffers in 100 ml of solution respectively, containing 3 mM-KH_2PO_4, 3 mM-$MgCl_2$ and 100 mM-KCl. The solution containing 3 mM-ATP was examined only at pH 7.5. These solutions were incrementally titrated with an $MnCl_2$ solution containing 10^{-6} mol of Mn^{2+}/ml. The ^{31}P line widths were determined as the width at half-height following a 10 min equilibration at 21 °C. Each spectrum required 2000–10000 scans depending on the concentration of Mn present in the solution. Repetition rates were 1.8 s, spectral widths 16000 and 8000 data points zero filled to 16000 were used. Little or no measurable change in line width could be made beyond 500 Hz.

RESULTS

Effect of Mn^{2+} on the spectra of maize root tips and sycamore cells under aerobic conditions

Fig. 1(*a*) depicts a representative ^{31}P spectrum of 700–900 maize root tips taken under the fast acquisition methods described earlier (Pfeffer *et al.*, 1986). The assignment for each resonance is given in the legend. The ratio of resonances in the low-field region of the spectrum, i.e. the sugar phosphates, cytoplasmic P_i and vacuole P_i, to the nucleotide resonance, i.e. NTP and uridine 5′-diphosphoglucose (UDPG), is distorted by a factor of 2:1 respectively because of the rapid recycling time (0.162 s delay between scans) used for acquisition of the spectra (Pfeffer *et al.*, 1986). Although this method underestimates the size of the vacuolar and cytoplasmic P_i pools relative to the nucleotides, it allows us to detect, with greater sensitivity, those resonances representing the nucleotides. This is especially important for study of Mn^{2+} line broadening in the maize root tip spectra, since extreme broadening of the nucleotide resonances is apparent immediately following introduction of Mn^{2+} into the perfusion medium. As indicated in the description of the sycamore cell spectra that follows, rapid recycling was not used (0.6 s pulse delay was used), since only very minor broadening of the nucleotide resonances was observed. Therefore, aside from the vacuole P_i resonance the detection of severely attenuated signals due to the presence of Mn^{2+} was not mandated.

The positions of cytoplasmic P_i (δ 3.01) and vacuolar P_i (δ 0.98) correspond to pH values of 7.8 and 5.5 respectively for these compartments in the first spectrum (Fig. 1*a*) taken at the initiation of the experiment with no Mn^{2+} present (Pfeffer *et al.*, 1986). We note that the vacuole resonance is relatively small (under slow acquisition conditions, i.e., at approx. 16 s recycle time it would

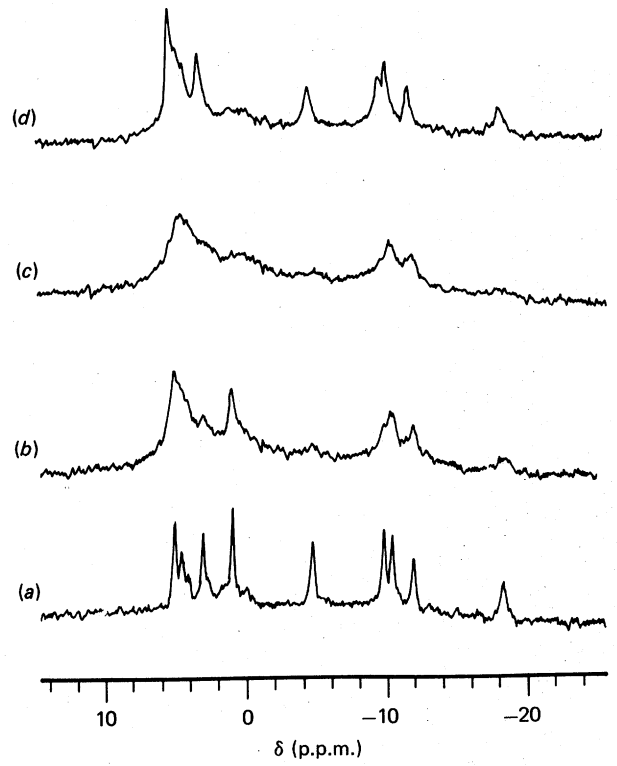

Fig. 1. Mn^{2+} migration and entrapment in the vacuole of maize root tips

161.8 MHz ^{31}P n.m.r. spectra, 10000 scans each (27 min), of maize root tips obtained during perfusion with 1 litre of oxygenated solution of 0.1 mM-$CaSO_4$, 50 mM-glucose and 10 mM-Mes buffer at pH 6.2: (*a*) following 3 h of perfusion, (*b*) the same as (*a*) except 0–30 min after the solution was made 1.0 mM in $MnCl_2$, (*c*) the same as (*b*) 1–1.5 h after $MnCl_2$ addition, (*d*) after perfusion with Mn^{2+}-free buffer as in (*a*) for 1.5–2 h. The resonance assignments are as follows: 5.10 δ sugar phosphates (principally glucose-6-phosphate); 3.01 δ cytoplasmic P_i; 0.98 δ vacuolar P_i; −4.80 δ γ-NTP; −9.5 δ α-NTP; −10.25 δ UDPG and NAD; −11.74 δ UDPG; −18.26 δ β-NTP.

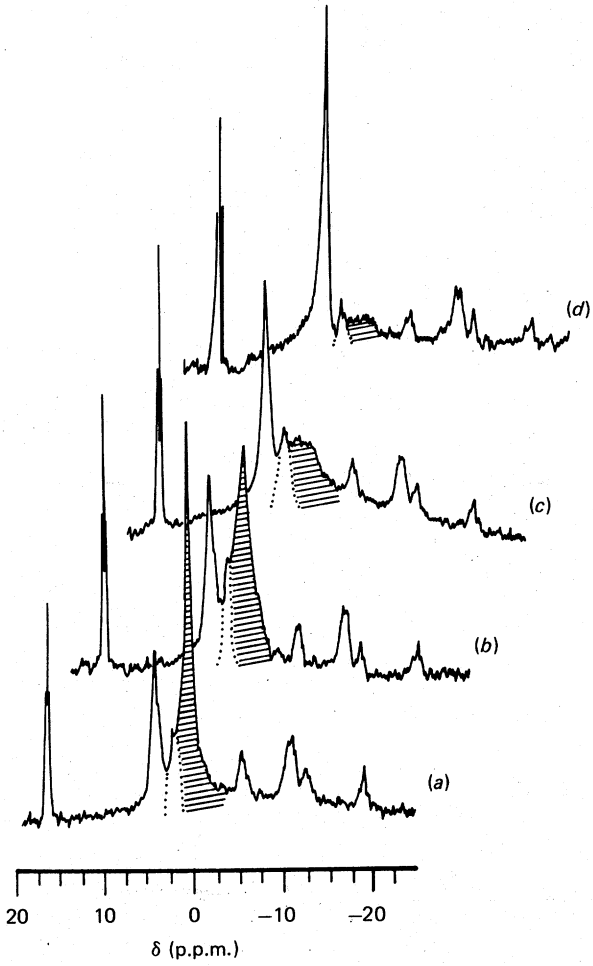

Fig. 2. Mn^{2+} migration and entrapment in the vacuole of sycamore cells

81.01 MHz ^{31}P n.m.r. spectra, 3000 scans each (30 min), of sycamore cells obtained during perfusion with 300 ml of oxygenated culture medium devoid of Mn and phosphate at pH 6.5: (*a*) following 2 h of perfusion, (*b*) the same as (*a*) except 30 min to 1 h after the solution was made 1.0 mM in $MnCl_2$, (*c*) the same as (*b*) 5–5.5 h after $MnCl_2$ addition, (*d*) after perfusion with Mn^{2+}-free buffer as in (*a*) for 2.5–3 h. The resonance assignments are as follows: 4.25 δ sugar phosphate, 2.18 δ cytoplasmic P_i, 0.23 δ vacuolar P_i, −5.52 δ γ-NTP, −10.26 δ α-NTP, −10.92 δ UDPG and NAD, −12.40 δ UDPG, −19.02 δ β-NTP.

be twice as intense relative to the nucleotides resonances) because the cytoplasm occupies about 30% of the volume of 3-day-old maize root tip cells (Reid *et al.*, 1985). Following perfusion during 30 min with 1 mM-$MnCl_2$ we observe a general broadening in the spectrum (Fig. 1*b*). Resonance lines corresponding to the nucleotides seen at δ −4.80 (γ-NTP), −9.50 (α-NTP) and −18.26 (β-NTP) have almost completely broadened out due to Mn^{2+} complexation. The resonance corresponding to cytoplasmic P_i has also broadened out significantly. The vacuole P_i resonance is still evident at δ 0.98, with slight broadening. Within 1.5 h (Fig. 1*c*) almost complete loss of the vacuole P_i signal was noted along with a more pronounced broadening of the sugar phosphate and UDPG/NAD resonance regions. Following a washout procedure with Mn^{2+}-free buffer for 2 h at pH 6.0, all but the resonance corresponding to vacuolar P_i was restored (Fig. 1*d*). However, a residual uniform line broadening of approx. 7–10 Hz was observed throughout the spectrum. The pH of the cytoplasm was observed to be 7.8 as in spectrum 1(*a*). The level of sugar phosphates, i.e., the combined areas of the resonances from 4.00 to 5.10 δ had increased by 30% over the initial value as seen in Fig. 1(*a*).

Fig. 2 shows the results of a comparable experiment carried out under aerobic conditions at pH 6.0 for sycamore cells challenged with a 1 mM-$MnCl_2$ solution. All the resonance assignments are the same as given in Fig. 1 except all the chemical shift positions were uniformly observed at 0.75 p.p.m. higher field owing to a difference in the reference standard used (see the Experimental section). Spectrum 2(*a*) shows that the cytoplasm and vacuole are being maintained at pH 7.5 and 5.7 respectively. The large area of the vacuole P_i peak relative to the nucleotides and other cytoplasmic compounds is due primarily to the morphology of the cells. That is, the volume of the vacuole occupies approx. 90%

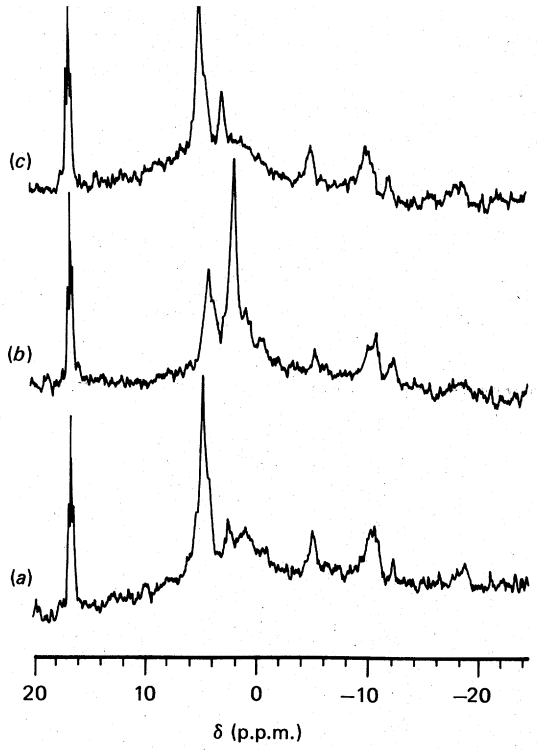

Fig. 3. Absence of Mn^{2+} leakage from the vacuole compartment of sycamore cells during anoxia

81.01 MHz ^{31}P n.m.r. spectra, 1000 scans each (10 min), of sycamore cells obtained: (*a*) as in Fig. 2(*d*), (*b*) during 30–40 min of anoxia (no perfusion), (*c*) following 0–10 min of aerobic treatment with perfusion.

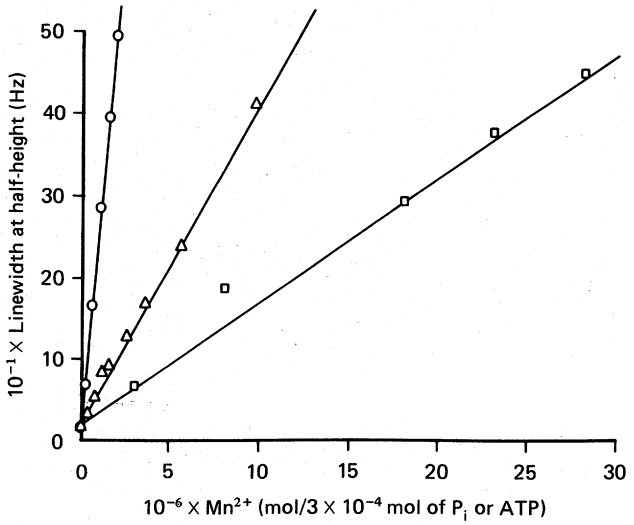

Fig. 4. ^{31}P n.m.r. line broadening in P_i and ATP is a function of pH and Mn^{2+} concentration

Titration plots showing the change in ^{31}P n.m.r. signal line width at half-height ($\delta_{\frac{1}{2}}$) for γ-ATP and P_i as a function of the number of mol of Mn^{2+} added to a solution containing 3×10^{-4} mol of ATP and P_i respectively. ○, P_i at pH 7.5, $P_i/Mn^{2+} = 150$; △, P_i at pH 5.5, $P_i/Mn^{2+} = 24$; □, ATP at pH 7.5, $ATP/Mn^{2+} = 10$.

of the cells (Roby *et al.*, 1987). Doubling of the pulse repetition time (0.6–1.2 s) did not appreciably alter the ratio of the resonances representing P_i and the nucleotides. The resolution achieved in these spectra are inferior to those given in Fig. 1 owing to the fact that they were obtained at one-half the magnetic field of the former. Following 30 min to 1 h of perfusion, (Fig. 2*b*) no evidence of nucleotide complexation was observed. However, significant Mn^{2+} penetration of the vacuole occurred, as seen by the severe line broadening and loss of 30% intensity of the vacuolar P_i resonance at 0.23 δ. Also, the original intensity of the cytoplasmic P_i resonance was still apparent even though the excessive broadening of the vacuolar P_i resonance obscured most of it. Based on the resonance positions of the cytoplasmic and vacuolar P_i peaks, the pH values remained at 7.5 and 5.7 respectively. Following 5–5.5 h of perfusion with the 1 mM-Mn^{2+} solution (Fig. 2*c*) almost complete loss of the vacuolar P_i resonance was noted, yet line widths of the nucleotides were essentially unchanged. In addition, the intensity of the sugar phosphate region (δ approx. 4.25) increased by 25% and the sharp resonance representing cytoplasmic P_i was clearly evident at δ 2.20. An additional 2.5–3 h of washing out the cells with Mn^{2+}-free buffer resulted in the spectrum seen in Fig. 2(*d*). Although there appears to be a shift in the remaining vacuolar P_i to the cytoplasm, there is also a significant 57% increase in sugar phosphate intensity as well as an approx. 15% loss of nucleotides and cytoplasmic P_i. All resonances except for vacuolar P_i have remained at their initial line widths. The pH of the cytoplasm as measured by the P_i resonance also remained at 7.5. Experiments carried out with 0.5 mM-Mn^{2+} show comparable results in which 1.0 mM-Mn^{2+} was used.

Effects of anoxia on sycamore cells containing vacuole-trapped Mn

Following the trapping and washout of Mn^{2+} from sycamore cells as described above, the cells were subjected to anoxia, whereby the perfusion system was stopped. Spectra, as shown in Fig. 3, were obtained in 10 min intervals to follow the rapid hydrolysis of sugar phosphate seen at δ 4.22. A drop in the cytoplasmic pH from 7.45, δ 2.15 (Fig. 3*a*) to 6.9, δ 1.61 (Fig. 3*b*) was noted after 30–40 min of anoxia. In addition, a dominance of cytoplasmic P_i was also evident as reported previously (Martin *et al.*, 1982). No estimation of vacuolar pH could be made owing to the absence of a measurable P_i resonance. The NTP signals dropped to near non-detectable levels. An aerobic state was re-established within 10 min of the resumption of aerated perfusion (Fig. 3*c*) as evidenced by a shift in the cytoplasmic P_i resonance to δ 2.05 (pH 7.35) at its near original intensity. No change was observed in the line widths of the resonances representing the cytoplasmic components following the sequential treatment of anoxia and aeration. NTP, P_i and glucose-6-phosphate signals were also almost restored to their original intensity.

Estimation of P_i/Mn^{2+} and ATP/Mn^{2+} ratios and ratios of Mn^{2+} in cytoplasmic and vacuolar compartments

In order to establish the minimum level of Mn^{2+} necessary to effect the observed line broadening of the P_i and NTP resonances *in vivo*, we titrated known concentrations of P_i at pH 5.5 and 7.5, and ATP at

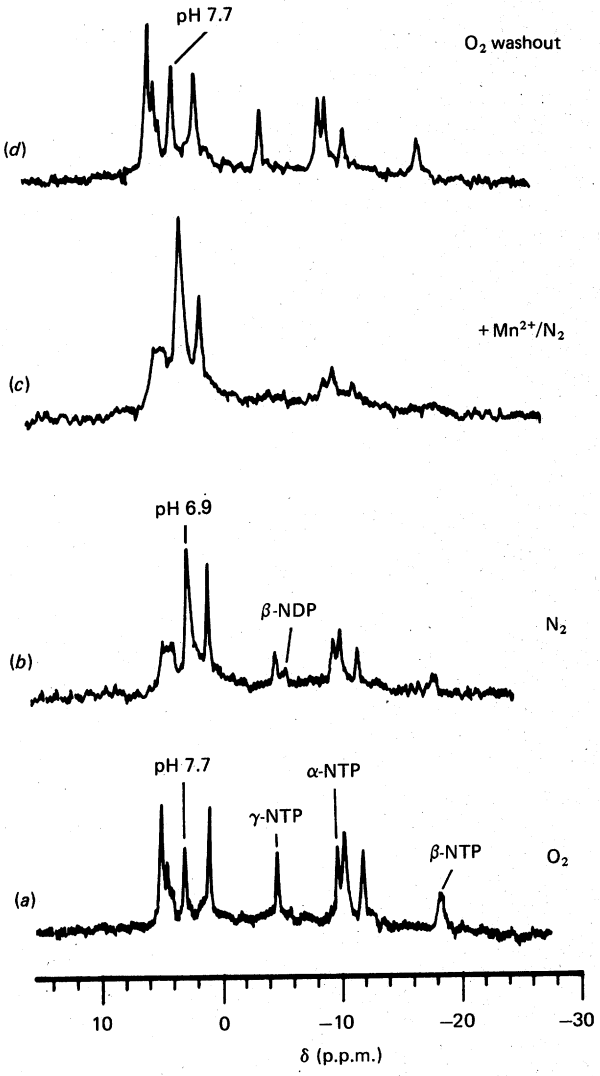

Fig. 5. Suppression of Mn^{2+} uptake in maize root tips during hypoxia

161.8 MHz ^{31}P n.m.r. spectra, 10000 scans each (27 min), of maize root tips obtained during perfusion with a 1 litre solution of 0.1 mM-$CaSO_4$/50 mM-glucose/10 mM-Mes buffer at pH 6.2: (*a*) following 2 h of perfusion under O_2, (*b*) during an additional 1–1.5 h of perfusion under N_2, (*c*) during an additional 1–1.5 h under N_2 in the presence of 1 mM-Mn^{2+}, (*d*) following 1.5–2 h washout with Mn^{2+}-free buffer as in (*a*) and O_2 bubbling (aerobic conditions).

pH 7.5 *in vitro* and measured the resulting ^{31}P n.m.r. line widths corresponding to those observed in the experiments *in vivo*. Fig. 4 shows a plot of mol of Mn^{2+} added to 3×10^{-4} mol of either P_i or ATP versus the resulting width at half-height of the corresponding P_i or γ-ATP resonance at pH 5.5 or 7.5. Based on the results of these plots, if we had equal amounts of P_i in both cytoplasm and vacuole, it would require 6.3 times as much Mn^{2+} to produce comparable P_i line broadening in the vacuole as in the cytoplasm. However, since we may also have a comparable concentration of NTP in the cytoplasm, whose binding constant is 158 times larger than that of P_i (Martin & Mariam, 1979), and which requires 15 times as much Mn^{2+} for full line broadening as does vacuolar P_i, the final distribution will require 2.5 times as much Mn^{2+} in the cytoplasm as the vacuole to produce the observed results. For the case of maize root tips, the ratio of vacuolar P_i to cytoplasmic P_i is 1.9 based on a quantitative comparison of ^{31}P peak areas (Pfeffer *et al.*, 1986). The coresponding amount of NTP is also 0.54 times the amount of vacuolar P_i in the ^{31}P spectrum of root tip tissue. From the ratios given in Fig. 4 we can calculate that it would require a minimum of approx. 1.4 times more Mn^{2+} in the cytoplasm than in the vacuole to produce the observed fully line broadened ^{31}P spectrum *in vivo*. In sycamore cells we have a somewhat different situation, since there is approx. 9 times as much P_i in the vacuole as in the cytoplasm, and the cytoplasmic P_i and NTP concentrations are approximately equivalent. Consequently, to satisfy the full complexation of vacuolar P_i would require that 3.5 times more Mn^{2+} be present in the vacuole than in the cytoplasm. Based on the estimate of approx. 1.0 mM-P_i and 1.0 mM-NTP in the cytoplasm and approx. a 1.0 mM concentration of P_i in the vacuole (Roby *et al.*, 1987) there should be a minimum concentration of 100 μM and 31 μM complexed Mn^{2+} in these compartments respectively to produce a fully line broadened ^{31}P spectrum.

Mn^{2+} movement into cells and tissue during hypoxia

Subjecting whole tissue or cells to an atmosphere of N_2 or Ar with continuous liquid perfusion and removal of local concentrations of CO_2 resulted in a state of hypoxia which is easily characterized by ^{31}P n.m.r. (Roberts *et al.*, 1984*b*; Pfeffer *et al.*, 1986). This stable anaerobic condition is attainable for periods of up to 25 h with little or no deleterious effects on the metabolic condition of the cells (Roberts *et al.*, 1985). Fig. 5(*a*) shows the spectrum of maize root tips under aerobic conditions. The high pH of the cytoplasm (7.7) is reflected in the position of the cytoplasmic P_i resonance at 3.03 δ. Following perfusion of the tissue with N_2-saturated perfusate for 1–1.5 h we observed significant changes in the ^{31}P spectrum reflecting a state of hypoxia (Fig. 5*b*). At this point the cytoplasmic pH has dropped to 6.9 as evidenced by the movement of the cytoplasmic P_i to 2.15 δ. In addition, breakdown of glucose-6-phosphate and loss ($\sim$ 30–40 %) of the nucleotide levels with a concomitant increase in cytoplasmic P_i was observed. NDP production (approx. 50 % of NTP) was also observed in this state as shown by the presence of the β-NDP peak at -5.75 δ and the additional peak area seen for the α-NDP resonance which coincided with the α-NTP resonance at -9.5 δ. Perfusion under a N_2 atmosphere for an additional 1–1.5 h in the presence of 1 mM-Mn^{2+} resulted in additional build-up of cytoplasmic P_i levels and line broadening of the NTP resonances (Fig. 5*c*). In separate experiments, efforts to remove Mn^{2+} through repeated washing of the tissue with perfusate devoid of Mn^{2+} under a N_2 atmosphere for 1.5–2 h did not regenerate the NTP resonances (results not shown). Re-introduction of O_2 into the washout medium for 1.5–2 h, however, restored the tissue to its initial aerobic state (pH of the cytoplasm 7.7) as seen in spectrum 5(*d*). In contrast with spectrum 1(*d*), little or no residual line broadening was noted. Also a 34 % increase in the size of the sugar phosphate region, predominantly glucose-6-phosphate, was observed relative to spectrum 5(*a*).

As noted earlier, the ^{31}P spectra of sycamore cells are characterized by their large vacuolar phosphate reson-

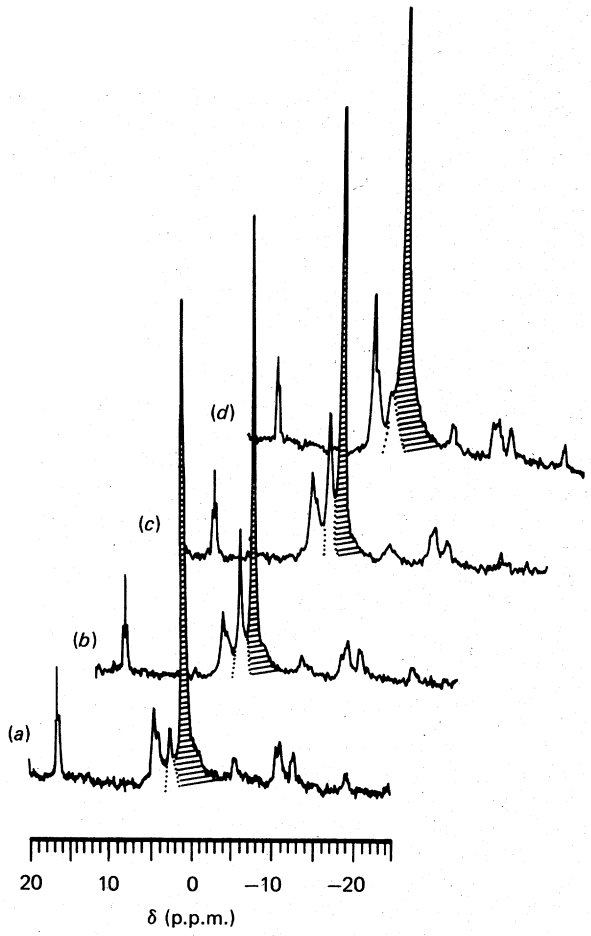

Fig. 6. Suppression of Mn^{2+} uptake in sycamore cells during hypoxia

81.01 MHz ^{31}P n.m.r. spectra, 3000 scans each (30 min), of sycamore cells obtained during perfusion with 300 ml of culture medium devoid of Mn and phosphate at pH 6.5: (*a*) following 1 h of perfusion under O_2, (*b*) the same as (*a*) except after 1–1.5 h of N_2 bubbling into the perfusate, (*c*) after an additional 1.5–2 h of hypoxia and perfusion with the above medium containing 1 mM-Mn^{2+}, (*d*) after 2–2.5 h of perfusion as in (*a*) under aerobic conditions (O_2 bubbling).

ance relative to all other resonances in the spectra, owing to the large difference in the volume of the vacuole and the cytoplasm (Roby *et al.*, 1987). Fig. 6(*a*) shows the ^{31}P spectrum of sycamore cells under aerobic conditions. The cytoplasmic pH as reflected by the position of its P_i resonance at 2.2 δ is 7.6. Introduction of N_2 gas into the perfusate for 1–1.5 h reduced the cytoplasmic pH to 7.0 (P_i 1.7 δ) and induced an increase in the cytoplasmic resonance by a factor of 2.5 (Fig. 6*b*). Whereas there was a 15–20 % decrease in the levels of NTP with a small increase in NDP (−6.50 δ), there was almost no change in the level of UDPG/NAD observed. After perfusion with the perfusate containing 1 mM-Mn^{2+} under a N_2 atmosphere for an additional 1.5–2 h we observed no significant difference in the signal intensity or line broadening of any of the nucleotide resonances (Fig. 6*c*). A comparable washout of the cells, as discussed above with Mn^{2+}-free medium under hypoxic conditions did not change this condition. Final restoration of the aerobic state yielded a spectrum (Fig. 6*d*) showing the same levels of nucleotides (line widths the same as in Fig. 6*a*) and a comparable cytoplasmic P_i level with a somewhat broadened vacuolar P_i resonance relative to that observed in the initial aerobic and hypoxic spectra. The initial line widths of the cytoplasmic and vacuolar P_i resonances throughout this experiment are shown by shading. With cytoplasmic pH readjusted to approx. 7.5, however, it is difficult to obtain an accurate measurement of the position of this resonance due to the broadened vacuolar P_i resonance. The sugar phosphate resonance region increased by 40 % over that observed in the initial spectrum 6(*a*).

DISCUSSION

It has been demonstrated that Mn^{2+} uptake in excised root tips under aerobic conditions occurs sequentially with a rapid build-up in the cytoplasm as indicated by the observed line broadening of the NTP resonances (Kime *et al.*, 1982; Pfeffer *et al.*, 1986). Weaker complexation of cytoplasmic UDPG/NAD and sugar phosphate resulted in only slight line broadening of their corresponding resonances. Following the complete line broadening ($\delta_{\frac{1}{2}} > 500$ Hz) of the cytoplasmic P_i resonance, further movement of Mn^{2+} across the tonoplast was manifested by the eventual suppression of the vacuolar P_i resonance. The inability to remove the Mn^{2+} from the vacuole by continuous washing with Mn^{2+}-free buffer is presumed to be characteristic of the detoxification or trapping properties associated with this non-metabolically active compartment (Foy, 1973). While the exact molecular mechanism of Mn^{2+} transport and compartmentation in sycamore cells and corn roots remains to be established, the observation mentioned in this study does provide the necessary information to formulate the energetic requirements of these processes.

Based on the inhibitory effect of Ca^{2+} on the transport and compartmentation of Mn^{2+} in corn root tips, we proposed a membrane carrier model for divalent cation transport (Pfeffer *et al.*, 1987*b*). In that model, we assume the influx of Mn^{2+} is modulated by the motion of charged carriers in the membranes. The membrane potential (negative, cytoplasmic side) across the plasma membrane would facilitate the inward transport of Mn^{2+} by increasing the concentration of the carrier at the outer surface. On the other hand, the entrapment of Mn^{2+} in the vacuole would suggest that the motion of the tonoplast membrane carrier could be limited by the acidic pH of the vacuole. As discussed in a previous report (Saris & Akerman, 1980), presumably these carriers can transport a variety of divalent cations. The sharing of carriers among some divalent cations in plant systems is supported by the discovery of a nH$^+$/Ca^{2+} antiporter which also exhibits transport ability of other divalent cations, in isolated plant tonoplast vesicles (Shumaker & Sze, 1985, 1986) and the observed nH$^+$/Ca^{2+} antiport movement in the plant plasma membrane (Rosi-Caldogno *et al.*, 1987). The P_i content in sycamore cell vacuoles is approx. 2.5–3 times (Figs. 1 and 2) that in the corn root tip cell vacuoles. The observation that complete suppression of the sycamore vacuolar P_i signal by paramagnetic Mn^{2+} required approx. 3 times as much time (1.5 h versus 5 h) as it required in maize root cells, indicates that the rates of movement of Mn^{2+} for the

overall process, i.e., final entrapment in the vacuole in both cell types appear to be comparable. Presumably the rate-determining step for final entry into the vacuole is the non-facilitated migration of Mn^{2+} across the tonoplast membrane. Since this relatively slow process is comparable in both cells it is clear that differences in the rates of facilitated movement of Mn^{2+} across the plasma membrane must be responsible for the divergent results. Accumulation of Mn^{2+} in the corn root tip cell cytoplasm indicates that the metal ion is passing through the plasma membrane more rapidly than it can be transported across the tonoplast. In contrast, the flow of Mn^{2+} through the sycamore cell plasma membrane and tonoplast, is well matched, i.e., a relatively slower uptake across the plasma membrane does not allow any significant concentration of Mn^{2+} to accumulate in the cytoplasm.

To account for the difference in migration rates across each plasma membrane we must consider possible differences in the Mn^{2+}-carrier binding affinities. It is well established that dicotyledon cells such as sycamore contain a relatively high concentration ($\sim 35\%$) of acidic polysaccharides within their cell walls (Prakash & Brinson, 1984), whereas monocotyledon cell walls such as those found in maize root tissue are low in these polymers and typically contain only a few per cent of these charged carbohydrates (Dever *et al.*, 1968). As a consequence of this composition difference one would anticipate that under comparable conditions the microenvironment of the cell walls adjacent to the plasma membrane would be more acidic in the sycamore cells. Consequently, a higher probability for protonation of the plasma membrane carrier protein would result in a lower affinity for metal ion binding and a lower rate of Mn^{2+} movement. In addition the greater ion-exchange properties of these cell walls would also lower the effective free metal ion concentration in proximity to the membrane surface. In the relatively more neutral environment of the maize cell wall, metal ions can compete more effectively for the plasma membrane carrier and so they can move with greater frequency into the cytoplasm.

Over long periods of washing sycamore cells with Mn^{2+}-free buffer under aerobic conditions, we have not observed movement of vacuole-trapped Mn^{2+} into the cytoplasm. During anoxia, i.e., when all circulation of perfusate and O_2 are shut off from the sycamore cells acidification of the cytoplasm and enhanced movement of protons from the vacuole to the cytoplasm occurs (Martin *et al.*, 1982). It was presumed that such a condition might also promote metal ion movement back from the vacuole to the cytoplasm. To examine this point we looked at the spectra of sycamore cells whose vacuoles were loaded with sequestered Mn^{2+}. During the limited period of anoxia (~ 40 min), no evidence of Mn^{2+} leakage into the cytoplasm could be detected by observable line broadening of the cytoplasmic resonances. This finding is consistent with the existence of a tonoplast membrane carrier which is highly protonated in the acidic environment created during anoxia (Schumaker & Sze, 1986).

When plant cells are subjected to the conditions of hypoxia (circulation of perfusate saturated with N_2), their cytoplasm undergoes acidification owing to accelerated glycolysis and decreased tricarboxylic acid-cycle activity. Subsequently this leads to the production of excess pyruvate and ultimate formation of lactate, ethanol and alanine (Pradet & Bomsel, 1978; Roberts *et al.*, 1984*a*; Fan *et al.*, 1986). Under these conditions maize root tips can be maintained for up to 25 h with little loss in viability (Pradet & Bomsel, 1978; Roberts *et al.*, 1985; Pfeffer *et al.*, 1986). The accelerated hydrolysis of glucose-6-phosphate results in a large build-up of P_i in the cytoplasm. However, the vacuolar P_i and pH remain relatively constant. Acidification of the cytoplasm to pH 6.9 has the effect of (1) lowering the transmembraneous proton potential and (2) promoting protonation of both the plasma membrane and tonoplast carriers at the cytoplasmic surfaces. This results in limiting the facilitated motion of the plasma membrane carrier and so metal ion uptake across the plasma membrane can only be accomplished by slow diffusion. In addition, transport across the tonoplast is highly suppressed because the protonated tonoplast carrier cannot readily release its protons into the already acidified environment of the cytoplasm.

Under anaerobic conditions as shown in Figs. 5 and 6, only trace quantities of Mn^{2+} were detectable in the cytoplasm of the sycamore cells. In contrast, the maize root tip cells showed full complexation of the nucleotides by cytoplasmic Mn^{2+} within a short time period. These results indicate that the slower Mn^{2+} plasma membrane migration that is observed under aerobic conditions is almost completely halted during hypoxia in sycamore cells. Since Mn^{2+} migration across the maize cell plasmalemma appears to be more rapid than across the sycamore cell plasmalemma under aerobic conditions one might anticipate that a similar difference in the rates of migration would be observed during hypoxia. The metal ion movement into the vacuole during hypoxia appears to be completely shut down for both cell types during the period of these experiments. This is not surprising since the non-facilitated movement of Mn^{2+} within the tonoplast is already relatively slow under aerobic conditions. The final spectrum (Fig. 6*d*) of the sycamore cells, showing a slightly broadened vacuole P_i resonance following Mn^{2+} washout under aerobic conditions, does, however, indicate that any trace quantitites of Mn^{2+} that penetrated the plasmalemma during hypoxia were picked up by the tonoplast carrier following the resumption of aerobic conditions.

Under aerobic conditions, and to a lesser extent during hypoxia, following perfusion with 1 mM-Mn^{2+} solutions and washout with Mn^{2+}-free buffer, we observed an increase in glucose-6-phosphate as seen by an increase in the glucose-6-phosphate resonance. As mentioned earlier, this increase is not associated with a shortening of ^{31}P relaxation resulting from trace quantities of Mn^{2+}, but does depend on the availability of exogenous glucose (Pfeffer *et al.*, 1986). It appears that the additional glucose-6-phosphate production may result from the possible Mn^{2+} interaction with the hexokinase system in the cytoplasm. Alternatively, Mn^{2+} might have a depressive effect on the utilization of glycolytic products following the production of glucose-6-phosphate. The very large increase in glucose-6-phosphate following uptake of Mn^{2+} under aerobic conditions, as opposed to that observed during hypoxia, is consistent with the greater influx of Mn^{2+} observed in the former experiment. We also observed that the cytoplasmic P_i content did not change significantly, whereas the vacuolar P_i level dropped following the introduction of Mn^{2+} and wash-

out. This indicates that phosphate was drawn from the vacuole (Rébeillé *et al.*, 1983) to maintain the cytoplasmic phosphorylation potential which was disturbed by the presence of excess Mn^{2+}.

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